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Novel Role of MBL/MASP-2 in Neuronal Autoimmunity and NeuroAIDS

A thesis submitted in partial satisfaction of the requirements for the degree
Master of Science

in

Biology

by

Jimin Lee

Committee in charge:

      Professor Kumud K. Singh, Chair
      Professor Michael David, Co-Chair
      Professor Ella Tour

2013

The thesis of Jimin Lee is approved, and it is acceptable in quality and form for

publication on microfilm and electronically:

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Co-Chair

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Chair

University of California, San Diego

2013

DEDICATION

This is dedicated to my family and friends, and Kumud Singh.

Thank you all for loving, supporting and encouraging throughout my journey.

EPIGRAPH

"Part of the strength of science is that it has tended to attract individuals who love

knowledge and the creation of it. ... Thus, it is the communication process which is at the

core of the vitality and integrity of science."

Philip Hauge Abelson

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ABSTRACT OF THE THESIS

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by

Jimin Lee

Master of Science in Biology

University of California, San Diego, 2013

Professor Kumud K. Singh, Chair

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Human immunodeficiency virus-1 (HIV-1) is a life-threatening virus which disrupts host immune system, and about half of the HIV-1 infected individuals develop neurocognitive impairment in brain. Neuronal autoimmunity is also observed in patients with HIV-1 infection, resulting in inflammation and damage in neurons and axons. Mannose binding lectin, MBL, plays important role in HIV by its direct binding of gp120, envelope protein present on the surface of HIV-1through lectin mediated complement activation pathway. MBL is also associated with initiation of autoimmunity

due to its involvement in clearance of apoptotic cells. This study focuses on determining

the role of MBL mediated complement pathway in the induction of neuronal

autoimmunity during HIV-1 infection of the brain. Overall, higher MBL/gp120 immune

complex deposition, and MBL/gp120 interaction with axonal damage marker amyloid

precursor protein (APP) in HIV encephalitis (HIVE) brain suggest MBL mediated

neuroinflammation and neuronal damage in HIVE. Increased immunoreactivity of

MBL/gp120 immune complex with myelin oligodendrocyte glycoprotein (MOG), and

myelin basic protein (MBP) suggest that these interactions potentially damage

oligodendrocytes and interfere with myelination in HIVE. Increased deposition of MBL

mediated immune complexes (C3d,C5b9,IgG) in HIVE suggests that deposition of these

aggregates may play a key role in axonal damage, demyelination and neuroinflammation

in HIV encephalitis. Presence of C4, C5, and MBL autoantibodies and higher

inflammatory cytokine response (IL-1β, IL-6, IL-2, and IL-8, Rantes,MIP-1β2, and

MCP-1)in the cerebrospinal fluid (CSF) of HIV-1 infected individuals with

neurocognitive impairment suggests that presence of MBL autoantibodies is associated

with proinflammatory cytokine immune response in HIV-1 related CNS impairment.

I:

Introduction

Human immunodeficiency virus-1 (HIV-1) is a life-threatening virus which interferes with body's immune system making HIV-1 infected individuals susceptible to other infections and diseases. Currently, World Health Organization (WHO) has reported that there are more than 34 million individuals infected worldwide, thus making HIV infections a pandemic disease (Mandal, 2009). As HIV infection progresses, it leads to the final stage, the acquired immunodeficiency syndrome (AIDS) which leads to further depression of immune system from HIV mediated killing of immune cells. Without treatment, the survival rate for AIDS is dramatically low, making it sixth leading cause of death worldwide (World Health Organization, 2011). Immunodeficiency due to HIV/AIDS is more dangerous as opportunistic infections such as bacterial diseases, viral diseases, and fungal diseases like tuberculosis, cytomegalovirus, and candidiasis would actually turn deadly . The most prominent problem of this serious and threatening disease is that although the research has been ongoing for last 30years, no cure or vaccines to completely eliminate the HIV virus have not been developed. Antiretroviral treatment reduces the risk of death and complications, but the medications are expensive and multiple side effects were observed in HIV/AIDS patients.

HIV-1 disrupts immune system by depleting key immune response cells called CD4+ T lymphocytes that help to activate body's immune response (Fauci et al., 1988). Therefore, while normal CD4 cell count ranges from 500 to1500 CD4, HIV-infected patients are considered normal if their CD4 count is above 500 and AIDS patients have lower than 200 CD4 cells. While HIV-1 is most well known for its effects on the immune system and progression to AIDS, it can also cause neurological disorders such as AIDS

dementia complex, encephalitis (neuroinflammation and neurodegeneration), leukoencephalothy, lymphoma, paraparesis, Tourette syndrome, myelopathy, and psychological disorders (Jaegar et al., 2012). This neuroinflammation, neurodegeneration and cognitive/motor impairment would lead to symptoms like loss of coordinative motor movements, loss of capacity of cognitive thinking and behavioral changes. It has been found that about half of HIV-1 infected individuals develop some type of HIV-1 related neurocognitive disorder, despite the fact that the anti-retroviral therapy (ART) reduces the viral load of HIV-1 in the body (Heaton et al., 2009).

While HIV-1 enters into the central nervous system in early infection, it cannot cross the blood brain barrier (BBB) by itself (Williams et al., 2002). HIV-1 is transported by its binding to CD4 T lymphocytes or through monocytes, which will then cross the blood brain barrier, and become HIV-1 infected macrophages as it enters from blood to tissue. Microglia cells, the glia cells that scavenge infectious pathogens in CNS, will fuse with HIV-1 infected macrophages and form multinucleated giant cells (MGCs). Then, MGCs will replicate these virus-infected cells to activate astrocytes to release the signaling molecules (cytokines) to enable HIV-1 infected cells to enter from blood to the brain (Benea et al., 2012). HIV-1 infected brain's damage extends to neurons through inflammation, demyelination, and oligodendrocytes (glial cells that are part of myelin that coats the axons) (Miura and Koyanagi, 2006). HIV encephalitis is an inflammation of brain, and is defined by spreading of these HIV-1 infected microglia fused with microglial cells, mono-and multi-nucleated giant cells, and by diffusing myelin damage and lymphocyte infiltrates. Although some of antiretroviral drugs lower the risk of severe

HIV-associated dementia, these could not completely treat brain damage by HIV since these drugs are not capable to penetrate through the BBB. The mechanism of HIV-1 mediated neuroinflammation and neurotoxicity is not well understood, thus making it more difficult to design effective therapy for HIV-1 associated neurocognitive impairment.

The CD4 T cells are not only associated with HIV-1 infection, but are also critical in autoimmune diseases (Dittel, 2008). Autoimmune diseases occur when the immune responsive cells attack the healthy cells, thus the treatment of the autoimmune disease includes immunosuppressive drugs to decrease the immune responses. While autoimmune diseases are caused by excessive activation of immune responses, HIV/AIDS leads to severe immunosuppression. It is interesting to note that autoimmune diseases such as anti-phospholipid syndrome, vasculitis, polymyositis, Graves' disease, and systemic lupus erythematous are found in HIV/AIDS infected individuals during the early stage of infection (Goddard et al., 2002). In the early stage of HIV infection, when there is normal to low CD4 cell count (400 to 200 per cubic millimeter of blood), individuals are more susceptible to autoimmune diseases, however, not susceptible during the HIV-1 stage with severe immunosuppression. When HIV/AIDS patients undergo antiretroviral therapy and CD4 cell count is restored back to normal (higher than 500 per cubic millimeter of blood), autoimmune disease manifests itself again (Goddard et al., 2002). One example of autoimmune neurological disorder such as acute disseminated encephalomyelitis is also found in HIV-1 infected brain.

Important mechanisms that prevent individuals from developing autoimmune

diseases are detecting and eliminating pathogens via phagocytosis; and complement activation is one of these major pathways of host innate immune response . Complement system is composed of about 30 soluble and cell associated factors, and it plays important role in immune response (Caroll, 2004). Complement system participates in both innate and adaptive immune response , where activated complement components facilitate the removal of damaged apoptotic cells, clear cellular debris, and kill and promote phagocytosis of pathogens (Botto et al., 1998; Kemper, 2007; Korb et al., 1997). However, excessive activation of the complement system would lead to increase in inflammatory response, eventually leading to tissue damage (Kemper, 2007). For example, complement activation has been found to promote demyelination through inflammatory response, leading to demyelinating disease like multiple sclerosis. Furthermore, unwarranted complement activation contributes to neurodegenerative disease like Alzheimer's disease, Huntington disease, and Parkinson's disease, due to abnormal complement protein aggregation and deposition (Rus et al., 2006).

Complement activation is initiated through three different cascades, classical, alternative, and lectin pathway (Figure 1). The classical pathway is antibody dependent pathway, whereas alternative and lectin pathway are antibody independent. Classical pathway is activated when complement protein C1q binds to antibodies that are already bound to surface of pathogen (Wallis et al., 2002). Alternative pathway gets activated when C3b, another complement protein which is most abundant and able to self-activate, forms complexes directly with pathogen surface. The lectin pathway uses soluble pattern recognition molecules, collectins (collagen containing C type lectins) to recognize

carbohydrates on the surface of the pathogens (Bowuman et al., 2006) (Figure 1).

Lectin mediated complement activation pathway is critical to understand since it plays important role in innate immunity. It involves soluble pattern recognition glycoprotein, mannose-binding lectin (MBL) which is synthesized in the liver and circulates in the bloodstream (Mangano et al., 2008). Our lab has also shown that MBL is expressed in major cell types in the brain (Singh et al., 2011). An oligomer of MBL is composed of N-terminal region, collagen-like domain, neck region, and calcium dependent carbohydrate recognition domain (CRD) at c-terminus (Figure 2) (Tsutsumi et al., 2005). When MBL circulates in the body, it can be in higher order structure like dimer, trimers, tetramers, pentamers and hexamers. These clusters of multiple CRD binding sites lead to effective complement activation (Tsutsumi et al., 2005).

Through carbohydrate recognition domain, MBL binds to the antigen, D-mannose, N-acetylglucosamine, and glucose residues exposed on the surface of pathogen/apoptotic/damaged tissue. Subsequently, MBL activates MBL associated serine proteases (MASP)-1,2, and 3 and activated MASP will cleave complement proteins C2 and C4, that in turn form another complement protein C3 convertase, C4b2a (Endo et al., 2006; Hajela et al., 2002). C4b2a will generate fragments like C3a and C3b; and C3b complement protein is involved in opsonization, enhancement of phagocytosis by forming membrane attack complex, and eventually lead to phagocytosis/lysis of pathogen (Figure 1) (Arnold et al., 2006; Pradhan et al., 2010).

While MBL is involved in recognizing foreign molecules and clearing of damaged/apoptotic cells, it is also associated with autoimmune diseases (Carroll et al.,

2001; Malhotra et al., 1995; Russell et al., 1998). The major cause of the autoimmunity is impaired apoptotic cell clearance, and MBL has been found to be involved in clearing these apoptotic cells (Pradhan et al., 2010). When a cell goes through the apoptosis, membrane carbohydrates will alter, leading to increased expression of glycoproteins (Pardhan et al., 2010). MBL will then, recognize these enriched glycoproteins using carbohydrate recognition domain and facilitate clearance via activation of complement activation. Another study has shown that both low and high levels of MBL are related to autoimmunity (Carroll et al., 2001; Ezekowitz et al., 2003). When there is decrease in MBL level due to presence of a *MBL2* gene genetic variant, clearance of apoptotic cells will be impaired, leading to autoimmune diseases like systemic lupus erythematosus (SLE) (Figure 4) (Garred et al., 2001). Furthermore, MBL deficient SLE patients had increased levels of autoantibodies against molecules that are associated with apoptotic cells (Seelen et al., 2003). High level of MBL and excessively activated complement activation would lead to tissue damage and subsequent increase in autoantibody formation, causing both autoimmunity and further tissue damage through inflammation (Figure 4) (Lee et al., 2006).

Additionally, MBL has shown to be associated with HIV-1. It has been found that trimeric complex of envelope glycoprotein 120 (gp120) is essential in mediating HIV-1's entrance to target cell (Sanders et al., 2008; Wyatt et al., 1998). When gp120 is produced from mammalian cells, it has dense high-mannose sugar residues on its surface, which prevents gp120 to be recognized by immune response (Wyatt et al., 1998). There have been multiple attempts against sugar residues on gp120 for therapy, however these

have not been successful. It is interesting to consider that MBL recognizes these mannose residues on the gp120 via its carbohydrate recognition domain. Consistent with MBL's role in complement activation, it has been found that MBL's interaction with gp120 will lead to opsonization of HIV-1 leading to enhanced spreading of the virus (Bouhlal et al., 2007; Eisen et al., 2008; Ji et al., 2005)

The central nervous system (CNS) is an important target of neurological impairment occurring in HIV encephalitis patients (Spector et al., 2010). Recently, Dr. Singh group has shown that increase in MBL expression was found in neurons, astrocytes, microglia, and oligodendrocytes of HIV encephalitis (HIVE) versus non-HIV encephalitis patients, and higher expression was associated with neuroinflammation and neurodegeneration in HIVE individuals (Singh et al., 2011).

Higher plasma levels of functional MBL were reported in demyelinating disease, multiple sclerosis (Kwok et al., 2011). Furthermore, current research had found increased levels of autoantibodies of MBL and complement proteins autoimmune disease like systemic lupus erythematosus; however, the significance of autoantibodies in disease progression has not been studied (Takahashi et al., 2004). My study is focused on finding the role of MBL/MASP-2 mediated complement activation in the induction of autoimmunity in the HIV-1 related neurodegeneraiton and neuronal damage. Overarching hypothesis is that MBL mediated complement activation will induce the formation of immune complexes, damage neuronal axons, myelin oligodendrocyte glycoprotein (MOG) and myelin basic protein (MBP) leading to generation of autoantigens and neuronal demyelination in HIV-1 infected brain.

To test my hypothesis, first, I will determine the expression of MBL along with the axonal damage marker, amyloid precursor protein in post-mortem brain tissue of HIV+ cases with/without HIV encephalitis. After confirming axonal damage in these HIV-1 infected brains, I will determine the expression of MBL with demyelination marker, myelin basic protein (MBP), and analyze samples to determine if MBL mediated complement activation is involved in demyelination in HIV-1 infected brain. Furthermore I will quantify the accumulation of autoantibodies against MBL, MASP-2, other complement proteins like C2, C3, C4, C5, and IgG and MBP. Additionally, cerebrospinal fluid (CSF) and plasma of HIV-1 infected individuals with or without neurocognitive impairment will be evaluated for these autoantibodies and proinflammatory cytokines to examine potential association of complement autoantibodies with neuroinflammaiton and neurocognitive impairment.

II:

MATERIALS AND METHODS

2.1 Characteristics of the post-mortem brain samples from HIV-1 infected individuals

35 post-mortem frontal cortex brain tissue samples were obtained from California NeuroAIDS Tissue Network (CNTN) at the UC San Diego HIV Neurobehavioral Research Center. CNTN is approved to utilize postmortem brain tissues of HIV infected individuals by UCSD institutional research board. Of 35 subjects, there were 15 HIV + HIV encephalitis individuals, 16 HIV+ non-HIV encephalitis individuals, and 3 HIV-healthy individuals. 29 out of 35 were male. The ethnicities of 35 subjects included 20 Whites, 11 Hispanic, 3 African Americans, and 1 Pacific Islander. Cause of death included acute bronchopneumonia(a severe inflammation of bronchioles), and/or septicemia, severe infections throughout the body in lung, abdomen or urinary tract. The median age by the time of the death for these subjects was 43 years old. Autopsy was performed within 24 to 36 hours of death. While 51% of subjects in this study had antemortem neuromedical and neuropsychological examinations, the remaining 49% of subjects were only enrolled from autopsy cohort. Among those with antemortem examinations, 48% subjects were on antiretroviral therapy at the time of their last assessment, and 74% had taken at least one antiretroviral drug in their life.

2.2. Characteristic of Plasma/CSF Samples obtained from HIV-1 infected individuals with or without neurocognitive impairment (NCI)

50 plasma and cerebrospinal fluid (CSF) samples were obtained from UC San Diego HIV Neurobehavioral Research Center. Of 50 subjects, 20 were HIV+ Neurocognitive Impairment + individuals, 20 were HIV+ Neurocognitive impairment -,

and 10 were HIV- healthy individuals. 88% of plasma and CSF samples matched. For plasma samples, 36 out of 50 were male. Of 50 patients,27 were Whites, 16 Hispanic, 3 African Americans, and 4 others. The median age of the plasma samples obtained was 41 years old. For CSF samples, there 33 were male and 17 were female. 28 of 50 subjects were White, 15 Hispanic, 3 African American, and 4 were others. Median age of samples obtained was 40 years. For plasma and CSF samples, 26 and 24 out of 50 subjects developed AIDS respectively.

2.3.1 Immunofluorescence studies of MBL and different markers in HIVE vs. non-HIVE cases

An earlier published protocol was followed (Singh et al., 2011). Briefly,

Paraffinized tissue sections were incubated at 60°C for 1 hour, washed with citrisolve clearing agent (Catalog # 22143975, Fischer Scientific) and rehydrated first 10 minutes in 50:50 citrisolve and then in decreasing concentrations of ethanol for 5 minutes each, 100%, 95%, 70%, 50%, 25%, and phosphate buffer saline, PBS subsequently. Then, permeabilization buffer (Catalog # PB001, Invitrogen) was added to sections for 10 minutes at room temperature. For epitope retrieval, tissue sections were flooded with 10mM sodium citrate buffer, pH 6.0 (Catalog# AP9003-500, Thermo Scientific) and heated for 10 minutes in a650W microwave oven, and cooled down to room temperature. Afterward, sections were washed with PBS for 20 minutes and non-specific sites were blocked in 5% normal goat serum with 5% bovine serum albumin (BSA) in PBS for 1 hour at room temperature.

Primary Antibodies, rabbit polyclonal anti-MBL2 (Catalog #HPA002027, Sigma-Aldrich), marker for demyelination rat monoclonal anti-myelin basic protein (MBP) (Catalog # ab7349, Abcam), mouse monoclonal anti-enveloped glycoprotein (GP120) (Catalog #SC 58147, Santa Cruz Biotechnologies Inc.), Goat polyclonal anti-glycoprotein GP120 (Catalog #SC17459, Santa Cruz Biotechnologies Inc.), anti-MASP2 (Catalog #SC17905, Santa Cruz Biotechnologies Inc.), marker of axonal damage goat polyclonal anti-Amyloid Precursor Protein (APP) (Catalog # Ab105122, Abcam),mouse monoclonal anti-myelin oligodendrocyte glycoprotein (MOG) for oligodendrocytes (Catalog # SC73330, Santa Cruz Biotechnology Inc.), and mouse monoclonal anti-microtubule associated protein-2 (MAP-2) cell marker for neurons (Catalog # SC74421, Santa Cruz Biotechnology Inc.) were diluted into 1:200 in PBS with 1% BSA and were incubated overnight at 4°C. Next day, with PBS, sections were washed for 20 minutes. Then, sections were incubated in the dark for 1 hour at room temperature with secondary antibodies such as donkey anti-rabbit IgG conjugate Alexa Fluor 488 (Catalog # A21206, Invitrogen) for probing MBL antibody; donkey anti-mouse IgG conjugate Alexa Fluor 568 (Catalog # A10037, Invitrogen) for probing GP120, MAP2 or MOG; donkey anti-goat IgG conjugate Alexa Fluor 647 (Catalog # A21447, Invitrogen) for probing either APP or GP120; and chicken anti-rat IgG conjugate Alexa Fluor 647 (Catalog # A21472, Invitrogen) for probing MBP were diluted 1:200 in PBS with 1% BSA. After incubation, sections were washed for 20 minutes with PBS, air-dried for at least an hour and Prolong Gold anti-fade reagent (Catalog # P36931, Invitrogen) containing DAPI (4', 6-diamidino-2-phenylindole) for detecting cell nuclei was added to tissue sections. By letting these sections to air dry at least 24-48 hours, sections were then analyzed by confocal

microscope. Control experiments were performed with primary or secondary antibody alone to ensure that there was no cross-reactivity between the antibodies.

2.3.2 Confocal Microscopy

Olympus FV1000 in UC San Diego Neuroscience Microscopy Facility was used for immunofluorescence and confocal microscopy. For DAPI (marker for nuclei), 358nm excitation and 461nm emission was used; for 488nm laser wavelength 293-538nm;for 543nm laser wavelength 548-628nm, and for 647nm laser wavelength 651-672 nm were used. All the images were captured using X100 lens and sequential plane mode with an average of 4 were used. For each immunostained section, pictures of 4 corner areas and 1 center in each section were taken.. Then, positive cells co-localized with MBL and respective cell markers were counted, and the percentages of positive cells co-localized with markers per number of cells present were counted and percent increase of expression from HIVE versus non-HIVE and 'p' value were calculated using the 2-tailed unpaired t-$test$.

2.4 Detection of Autoantibodies: The ProtoPlex^{TM} Immune Response Assay

The ProtoPlex^{TM} Immune Response Assay is a bead-based multiplexed solution assay, similar to an ELISA. This assay uses internally dyed polystyrene beads with red and infrared fluorophores of different intensities. Each bead is given a unique number, or bead region, and has a unique emission wavelength, allowing differentiation of each antigen-bound bead. All the reagents and beads were specifically designed and provided by ProtoPlex Immune Response Assay Kit (Life Technology, Carlsbad, CA). C2, C3, C4,

C5, MBL, MASP2, Human IgG, anti-human IgG, and BSA magnetic beads were diluted

into Streptavidin HRP diluent (Life Technology, Carlsbad, CA) to have 5,000 beads per

well and 40,000 beads per region. Plate wells (Catalog # 90565163, Bioexpress,

Kaysville, UT) were coated with 100uL of multiplex bead mix. While preparing and

dispensing bead-plex to wells, multiplex beads were vortexed and sonicated routinely to

inhibit bead setting and aggregation. Then, 96-well plate (Black Microplates

FLUOTRAC 200 Medium Binding 40, Catalog # T-3026-17, BioExpress) was placed on

the Lifesep Magnet (Catalog # A14179, Life Technologies) for at least 1 minute and

supernatant was removed. Brain tissue lysate protein was prepared from HIVE, non-

HIVE, and HIV- brain tissues, and proteins were extracted via Qproteome Mammalian

Protein Prep Kit (Catalog # 37901, Qiagen, Valencia, CA). Matching plasma and CSF

samples of HIVE, non-HIVE, and HIV- were also obtained from California NeuroAIDS

Tissue Network, and brain tissue lysate, plasma, CSF samples were diluted 1:10, 1:200,

and 1:8 accordingly in Streptavidin HRP Diluent. Each sample was centrifuged 10

minutes at 4^oC to remove the debris. 100uL triplicates of each sample and negative

control (Streptavidin HRP Diluent) were added to plate and incubated for 1 hour at

700RPM in the dark at room temperature,. After incubation, plates were washed 3 times

with Wash Solution (Invitrogen) in water. For every wash, plates were placed on Lifesep

magnet for at least one minute and wash solution was removed. Anti-human IgG diluted

1:200 in Streptavidin HRP Diluent were added to each well and incubated in room

temperature for 1hr at 700 RPM. After incubation, beads were washed again with Wash

Solution 3 times; and 150uL of Wash solution was added to each well and incubated for 5

minutes at 700 RPM and loaded to MAGPIX analyzer. Each protein was designated to

different bead region. Luminex instrument, MAGPIX excited green LED 525nm and LED 635nm, to detect autoantibody present depending on the bead regions. Average of median fluorescent intensity (MFI) was obtained for analysis and *P* value was calculated using the 2-tailed unpaired *t-test*.

2.5 Inflammatory Immune Response Cytokine Assay

Human Cytokine Magnetic 25-Plex Panel Kit was used for this assay(Catalog # LHC0009M, Invitrogen). First, Antibody Bead Solution (Lot # 910514, Invitrogen) was sonicated and vortexed immediately prior to use, and magnetic bead solution was added into each well. Then, these magnetic beads were soaked with Working Wash Solutions (diluted 1:20 in water) and plates were washed two times. Afterwards, incubation buffer was added to each well, and to designated wells for standard curve: 0, 1:1, 1:2, 1:4, 1:8, 1:16, 1:32 diluted standards were prepared by combining Human 14-plex Standard (Lot # 836090, Invitrogen) and Human 16-Plex Standard (Lot # 891415, Invitrogen). To the wells designated for the samples, Assay Diluent and sample were added according to the dilutions and brought to 50uL volume. For plasma s, samples were diluted 1:4; and for CSF, samples were diluted 1:4 and 1:2. Negative control of Assay Diluent only was also added to designate wells. All the standards and samples were used as duplicates. The plates were then incubated for 2 hours at room temperature on the plate shaker (600rpm), washed with 200uL of Working Wash Solution two times. Then, 1: 10 diluted Biotinylated Detector Antibody Concentrate (Lot # 910515, Invitrogen) in Biotin diluent was added to each well, and incubated for 1 hour at room temperature on the plate shaker (600rpm). After a 1-hour incubation, 1:10 diluted detection antibody, Streptavidin-RPE

diluted in Streptavidin diluent were added and incubated for 30 minutes at room temperature on the plate shaker (600rpm). Afterwards, plates were washed 3 times, and 125uL of Working Wash Solution was added and incubated for 2-3 minutes on the plate shaker (600rpm) to re-suspend the beads. Plates were analyzed in MAGPIX instrument . Average of Median Fluorescent Intensity (MFI) was obtained for analysis and *P* value was calculated using the 2-tailed unpaired *t-test.*

III:

Results

3.1 MBL immunoreactivity is present in association with neurons and oligodendrocytes in post-mortem brain tissues with HIV encephalitis.

To determine the correlation of MBL expression with neuronal axons in the risk of encephalitis, frontal cortex brain tissue samples from HIVE and non-HIVE cases were immunostained with MBL antibodies. Antibodies against MBL and brain cell markers for oligodenrocytes (myelin oligodendrocyte glycoprotein, MOG), and neurons (microtubule-associated protein-2, MAP-2) were used to show the presence of MBL in the cells of HIV-1 infected brain. A co-localization of MBL with neuronal marker MAP-2 and oligodendrocyte marker MOG was found, showing MBL's interaction with neurons and oligodendrocytes (Figure 5). Additionally, DAPI stain clearly marked the nuclear DNA, showing that intact cells were studied.

3.2 Increased MBL expression in HIV encephalitis compared to non-HIVE, healthy HIV negative post-mortem brain tissue

Comparison of MBL expression in brain tissue of HIV encephalitis versus the non-HIVE subjects was done. Overall, there were38% to 45% higher MBL positive brain cells in HIVE to non-HIVE cases (n= 8, n=7 respectively, p <0.01, paired t-test) (Figure 4a). Frontal cortex brain tissues from HIV negative, healthy individuals did not show any significant MBL expression (Figure 4a).

3.3 Increased MBL expression and co-localization with viral gp120 in HIV encephalitis vs. non-HIVE post-mortem brain tissue.

Since MBL directly interact with mannose residues on the gp120 protein and form

immune complex, higher interaction of MBL with mannose residues on gp120 protein in HIV encephalitis was expected, and we found 19% increase in gp120 in HIVE vs. non-HIVE brain (p value <0.11, n=6 for HIVE, n=7 for non-HIVE) (Figure 4b). There was 31% increase in MBL co-localized with gp120in HIVE vs. non-HIVE brain (p value <0.21, n=6 for HIVE, n=7 for non-HIVE) (Figure 4c) and 16% increase in MBL co-localized with gp120 in oligodendrocytes in HIVE vs. non-HIVE cases (p value<0.3, n=4 for HIVE, n=4 for non-HIVE) (Figure 5a). Increase in MBL co-localization with gp120 indicates higher MBL association with viral proteins in HIVE. No gp120 protein was found in healthy HIV negative cases as expected (Figure 4b,4c,5a,7).

3.4 MBL Immunoreactivity and Co-localization with Marker of Axonal Damage

In order to investigate MBL's association with axonal damage, amyloid precursor protein (APP) was used in HIV-1 infected brain (Giometto et al., 1997; Gentlemen et al., 1993). In HIVE brain tissue samples, there was 1.62 folds increase in APP deposition as compared to non-HIVE brain (p value<0.01, n=7 for HIVE, n=6 for non-HIVE) (Figure 4d).Furthermore, MBL's immunoreactivity with APP was 1.76 folds higher in HIVE brains samples compared to non-HIVE samples (p<0.01, n=7,6 respectively)(Figure 4e).In neuronal cells, MBL co-localization with APP showed 1.73 folds increase (p<0.01, n=4 for HIVE and non-HIVE each), whereas in oligodendrocytes, 1.43 folds increase was found in HIVE versus non-HIVE brain tissue samples (p<0.1, n=5 for HIVE n=5 non-HIVE) (Figure 5b,5c,8). These findings suggest MBL is potentially involved in axonal damage and oligodendrocyte injury in HIV-encephalitis.

3.5. MBL immunoreactivity with myelin basic protein (MBP)

To first assess if MBL was associated with myelin sheath and demyelination, MBL co-localization with myelin basic protein MBP was determined in HIV-1 infected brain. In both HIVE and non-HIVE brain tissue samples (n=6 for HIVE, n=8 for non-HIVE), MBL co-localization with MBP was observed (Figure 6a). There was higher MBL association with myelin/neurons in HIVE, there was 28% increase in HIVE vs. non-HIVE (p value<0.4, n=4 for HIVE n=4 for non-HIVE) (Figure 6b). Then, to check MBL's immune complex association with myelin, MBL's immunoreactivity with gp120 and MBP was detected. There was 9% higher interaction of MBL with gp120 and MBP in HIVE than non-HIVE, thus showing that these immune complexes may induce interaction of MBL with myelin sheath in HIVE cases (p value<0.8 n=4 for HIVE n=4 non-HIVE) (Figure 6c). Moreover, it is shown that MBL co-localize with MBP, and also with MAP-2 and gp120, suggesting that MBL might be associated with myelin damage/demyelination occurring in HIV encephalitis (Figure 9).

3.6. Increased co-localization of MBL andMASP-2 in HIVE.

To investigate if MBL mediated complement activation induces excessive lectin complement activation in HIVE, MBL's immunoreactivity with MASP-2 was observed. MBL and MASP-2 interaction was significantly increased in HIVE compared to non-HIVE cases suggesting a higher activation of lectin pathway was found in HIVE individuals (Figure 7a).

3.7.Increased Deposition of MBL, MASP-2 and C3d, and C5b9 immune-complexes in HIVE Brain.

MBL/MASP-2 immunoreactivity with complement proteins involved in immune response was evaluated to analyze immune complex formation in HIVE. Significant increase in co-localizations of MBL, MASP-2, and C3d and MBL, MASP-2, and C5b9 were found in HIVE (Figure 7b,8a). This co-localization suggests MBL's association with immune complex deposition, and its higher expression suggests formation of immune complexes' association with neuroinflammation/neuronal damage in HIVE.

3.8. Increased Expression and Deposition of MBL and IgG Immune Complexes in HIVE Brain.

Immunoglobulin G protein, IgG, is the most common protein deposited in tissue and released due immune complex formation. Higher expression of MBL co-localized with IgG in HIVE suggests increased in MBL and IgG immune complex formation in HIVE in response to neuronal damage.

3.9. Immune response assay for detecting autoantibodies against MBL s, and Myelin Basic Protein (MBP).

Autoantibodies were detected from brain tissue lysate, plasma, and CSF samples from HIV-1 infected individuals with or without NCI. No significant autoantibodies were found in brain tissue lysate samples. HIV- samples were used as negative controls, and the average MFI was very low. In plasma, there was 16% increase in average net MFI(p value< 0.33) of C5 autoantibodies in HIV+ neurocognitive impairment (n=20) compared to HIV+ non-neurocognitive impairment patients (n=20) (Figure 10). In CSF samples, there were 13% increase in C4 autoantibodies (p value< .01), 17% in C5 autoantibodies

(p value<.19), 13% in MBL autoantibodies (p value<.07) of HIV+ neurocognitive impairment positive individuals versus neurocognitive impairment negative individuals (n=19 for HIV+ NCI+, n=20 for HIV+ NCI-) (Figure 11).

Elevated levels of autoantibodies of complement proteins C4, C5, and MBL suggest that MBL mediated complement activation in HIV-1 infected brain may induce generation of autoantigen leading to inflammation in neurocognitive impairment in HIV-1 infected individuals.

4.0.Cytokine Immune Response Assay for detecting MBL mediated inflammatory immune response in HIV-1 Infected individuals with or without neurocognitive impairment.

Cytokine immune response assay was done for 20 HIV+ with neurocognitive impairment (NCI+), 20 HIV+ without neurocognitive impairment (NCI-), and 10 HIV-healthy individuals' plasma and CSF samples. Cytokine concentrations were compared between NCI+, NCI-; and HIV- healthy individuals. 41% higher IL-8levels (p value<.04 n=11 for NCI+ n=15 for NCI-) for NPI+ vs. NPI- (Figure 12)were found in plasma samples. In CSF samples, 100% higher IL-1β (p value<. 07 n=10 for NCI+ n=13 for NCI-), 50% higherIL-6, (p value<. 06 n=17 for NCI+ n=20 for NCI-), 88% higherIL-2 (p value<. 05 n=11 for NCI+ n=12 for NCI-), and 24% higher IL-8 (p value<. 27 n=17 for NCI+ n=20 for NCI-) levels were observed (Figure 12). Higher levelsof chemokines were also obtained in NPI+ CSF samples, 44% higher for RANTES(p value<.1 n=10 for NCI+ n=11 for NCI-), 67% higher forMIP-1β2 (p value<.45 n=11 for NCI+ n=13 for NCI-), and 18% higher forMCP-1 (p value<.29 n=19 for NCI+ n=20 for NCI-) (Figure 12).

Overall, higher levels of cytokines/chemokines were observed in NCI+ patients.

IV:

DISCUSSION

Complement activation is an important mechanism of innate immunity, which is involved in removal of apoptotic cells, clearance of cellular debris, and phagocytosis of pathogens. Almost all complement proteins are expressed in brain and have been implicated in neuroinflammation and neuronal diseases. Excessive activation of the complement increases inflammatory response, leading to tissue damage, demyelination and neurodegenerative disease due to abnormal complement aggregation and deposition (Kemper et al., 2007; Rus et al., 2006).

Mannose binding lectin, a key molecule that plays a role in complement activation mediated by lectin pathway, facilitate clearance of apoptotic cell by recognizing glycoproteins expressed on the pathogens through its carbohydrate recognition domain. MBL2 RNA and proteins are also found in brain, and its level of expression is associated with neuroinflammation and neurodegeneration (Singh et al., 2011; Yanai et al., 2005). Thus, investigating MBL mediated complement activation is important in relation to HIV-1 infected brain, since MBL levels increase as HIV-1 disease progresses; and MBL can directly bind to HIV-1 through its carbohydrate recognition domain on the glycosylated mannose residues on gp120 proteins (Ji et al., 2005).

First, MBL's immunoreactivity was observed in HIV encephalitis and non-HIV encephalitis individuals' post-mortem brain tissues. The increase in MBL expression in HIVE suggests MBL mediated complement activation in HIV-1 infected brain (Figure 4a). This is consistent with Singh group's study, showing MBL's possible association with neuroinflammation and neurodegeneration in HIVE patients (Singh et al., 2011).

The presence of gp120 in brain (internalized in neurons) may also lead to neuroinflammation and neuronal death, apoptosis (Guha et al., 2012; Wyatt et al., 1998). Therefore, expression and co-localized MBL and gp120 were studied.. Overall, there was increase in gp120 expression and MBL co-localized with gp120 showing increased immune complex formation in HIVE (Figure 4b, 4c,9). Complement activation mediated by MBL and gp120 leads to opsonization of HIV leading to enhanced spreading of virus, thus the data indicates that there was higher activation of innate immune response in HIV encephalitis patients, due to binding of MBL to gp120, eventually leading to increased neuroinflammation/neuronal degeneration.

Additionally, MBL's immunoreactivity was observed in association with neurons and oligodendrocytes in post-mortem brain tissues with HIV encephalitis, to determine axonal damage and neuronal injury in HIVE individuals. Amyloid precursor protein is abundantly expressed in mammalian brain and its expression is increased when there is a traumatic brain injury, results in beta-amyloid deposits in Alzheimer's diseases (Giometto et al., 1997; Zheng and Koo 2006). Thus, APP is used in multiple studies as a marker of axonal damage. In our studies, we found that MBL was directly associated with beta-amyloid preferentially through its N-terminal cysteine rich domain (Larvie et al., 2012). Thus, an increase in APP and its co-localization with MBL HIV encephalitis brain shows that MBL-APP complexes are associated with induction of axonal damage in HIVE (Figure 5de). Moreover, MBL co-localization with APP and neuronal cell marker MAP-2 was higher in HIVE vs. non-HIVE cases (Figure 6a). This suggests that MBL potentially mediates HIV-1 related axonal in HIVE.

While functional MBL has been found to be increased in demyelinating disease like multiple sclerosis (Kwok et al., 2011), and demyelination has been found in HIV encephalitis, the role of MBL mediated complement activation in association with demyelination in HIV-1 has not been studied. Oligodendrocytes are the glial cells responsible for the formation and maintenance of myelin that coats the axons. MOG (myelin oligodendrocyte glycoprotein), a marker for oligodendrocytes, is exclusively expressed in central nervous system, and on the external layer of myelin sheaths (Mantegazza et al., 2004). Since there is evidence that oligodendrocyte are direct target of HIV-1 mediated damage and higher MOG antibody were observed in HIV-1 associated neurocognitive disorder, MBL's interaction with MOG was evaluated (Cosenza et al., 2002; Lackner et al., 2010). The increase in co-localization of MBL and APP in oligodendrocyte was found in HIVE vs. non-HIVE brain (Figure 6b), suggesting that MBL is potentially associated with myelin damage in HIVE. Furthermore, increased immune complex formation of MBL and gp120 in oligodendrocyte was found in HIVE (Figure 6c,8)implying that MBL-gp120 -interaction potentially damage oligodendrocytes and myelination in HIVE brain.

In HIV-1 infected individuals, neurological syndrome like demyelination is found, and studies show that most significant component that is released during demyelination is myelin basic protein (MBP) (Liuzzi et al., 1992; Mastroianini et al., 1990). While MBP is known to activate the complement pathway through classical and alternative pathway mechanisms, however, no study has evaluated its damage and interactions during lectin mediated complement activation. To assess if MBL interacts with MBP, MBL's co-

localization with MBP was evaluated, and as expected, MBL and MBP co-localization was found in both HIVE and non-HIVE brain (Figure 7a). An increase in MBL association with myelin/neuronal cell (Figure 7b) was observed in HIVE, showing that MBL interacts with neurons and myelin in HIVE. Since the immune complex of MBL and gp120 were presented along with MBP in HIVE (Figure 7c,8), MBL-gp120 mediated complement activation may be involved in myelin damage and demyelination through HIV-1 opsonization.

Studies have shown that over-activation of immune response leads to autoimmunity, inflammation, axonal damage, and demyelination in HIV-1 infected brain, and MBL is found to be associated with over-activation via lectin mediated complement activation (Heaton et al., 1009; Jacewicz, 2008; Singh et al., 2011). In HIVE, there was higher co-localization of MBL with its activated mannose binding serine protease, MASP-2, showing excessive activation of complement pathway. Furthermore, MBL/MASP-2 co-localization with complement proteins such as C3d and C5b was observed for immune complex formation, and there was significant increase in MBL,MASP-2,C3d and MBL,MASP-2,C5b immune complex deposition in HIVE. C3d is a fragment of complement protein C3 that is involved in reducing accessibility of viral protein through binding to gp120, and enhancing immune response (Topanta and Ross, 2006). Furthermore, as a component of membrane attack complex, an end product of complement activation for immune response, C5 is involved in phagocytosis and lysis of pathogen. Higher co-localization of MBL,MASP-2,C3-d and MBL,MASP-2, C5b in HIVE suggest MBL/MASP-2 mediated complement activation and immune complex

deposition in HIVE, could induce neuroinflammation and neuronal damage in HIVE.

Immunoglobulin G (IgG) antibodies are the smallest yet, most common and key protein involved in immune response. IgG positive immunoglobulin containing cells have been found in inflammatory disorders such as multiple sclerosis and HIV encephalitis. It is the most common protein observed in immune complex formation. Thus, immune complex formation between MBL and IgG was studied to link not only the immune complex deposition but also inflammation in HIVE. There was higher expression of MBL co-localized with IgG in HIVE suggesting increased MBL and IgG immune complex formation in HIVE in response to neuronal damage, and also leading to inflammation in HIVE. The deposition of MBL-mediated C3d, C5b9 and IgG immune complexes in HIVE indicates that it plays a key role in axonal damage, neuroinflammation, and neurocognitive impairment in HIV-1 infected individuals.

HIV-1 invades central nervous system during early stage of infection via infiltrate monocytes and lymphocytes, and HIV-1 has been found to be involved in release of proinflammatory cytokines/chemokines and viral proteins in HIV-1 (Guha et al., 2012). Individuals infected with HIV-1 have higher levels of chemokine producing cells, and positive correlation with viral load and levels of neruoinflammatory cytokine via the mechanisms utilizing HIV-1 envelope protein gp120 (Kelder et al., 1998; Lipton, 1996; Ramalingam et al.,2008; Zheng et al., 2008). MBL has been also reported to induce cytokine production in humans, yet not well studied in HIV-1 infected patients with neurocognitive impairment or neroinflammation (Chaka et al., 1997; Ghezzi et al., 1998). MBL mediated proinflammatory cytokine response and neuroinflammation was

evaluated to correlate lectin pathway and inflammation in plasma and CSF of HIV-1 infected patients with/without neurocognitive disorder. Overall, there was higher expression of inflammatory immune response cytokines, such asIL-1β, IL-6, IL-2, and IL-8, RANTES, MIP-1β2, and MCP-1 in HIV+ subjects with neurocogntivie impairment.Cytokine molecules like IL-2 and IL-8 are invovled in enhancing immune response by helping T cell immunologic memory, and induction of chemotaxis. IL-1β,MCP-1, RANTES are known to be responsible for the inflammatory response. Interleukin 8 (IL-8) is a chemokine produced by macrophages, and it is proinflammatory cytokine that enhance the immune response by induction of chemotaxis of cells, thus mediating migration of immune responsive cells toward the target cells. While IL-8 is often found in infectious/inflammation disease, it is known to increase transmission of HIV-1 (Narimatsu et al., 2005).HIV-1 gp120 mediates the increase in IL-8 poduction via nuclear factor kappa light chaIn enchancer of activated B cells, (NF-κB pathway)(Shah and Kumar, 2010). Thus, increase in IL-8 suggests an increase in viral load and neuroinflammation in HIV+ NCI+ patients. While IL-1β is found to lead transcription of proinflammatory cytokines like TNF-α, IL-6 and interferon (IFN), RANTES is found to help particular cytokines such as IL-2 (Guha et al., 2012). In monocytes and macrophages, complement proteins such as C3a and C5a have been shown to induce the synthesis of pro-inflammatory cytokines like TNF-a, IL-1β, IL-6, and IL-8 and in HIV-1-induced neuronal diseases, higher levels of TNF-α, IL-1β, IL-6, IL-8, monocyte chemoattractant protein-1 (MCP-1) *in vivo* and *in vitro* (Kelder et al., 1998; Stahel et al., 1998). While IL-2 production is enhanced by MBL,IL-6 is is secreated by macrophages in response to mannose conating pathogen, i.e. after pathogen recognitionby the MBL, the increase in

these cytokinesindicate that MBL is associated with pro-inflammatory cytokine immune response in HIV+ neurocogntive impairment patients leading to neuroinflammation and neuronal damage.

To investigate if MBL-mediated complement activation is involved in neuronal autoimmunity and induction of autoantigen, autoantibody detection assay were performed. Autoimmune neurological disorder such as acute disseminated encephalomyelitis is found in HIV-1 infected brain and higher autoantibodies against proteins involved in clearance of apoptotic cells including MBL were found in autoimmune diseases (Jacewiz, 2008; Yehuda et al., 2007). Although still not well studied, autoantibodies were found to be associated with neuronal damage when there was impaired clearance of apoptotic cells; these apoptotic cells induce production of autoantibody leading to inflammatory reaction (Fishelson et al., 2001). Since inflammation and demyelination have been found in brains of HIVE, and HIV+ neurocognitive impairment patients, autoantibodies against complement proteins, MBL/MASP-2, and MBP were evaluated. Overall, increased autoantibodies against complement proteins C4, C5, and MBL were found in HIV-1 infected neurocognitive impairment patients (Figure 9,10). When MBL mediated complement pathway cleaves C2 and C4 complement proteins, it leads to activation of C3 convertase to activate C5 to form membrane attack complex for phagocytosis/lysis of pathogen. The increase in autoantibodies against proteins involved in lectin pathway and MBP in HIV-1 neurocognitive impairment patients suggest that MBL mediated complement activation leads to potential neuronal autoimmunity and demyelination in HIV-1 infected brain.

The working model of this study shows that when there is HIV infection, there is increase in MBL expression, leading to excessive complement activation pathway, autoantibody generation, and immune complex deposition. These would result in aggressive immune response, autoimmunity, tissue damage, axonal damage, demyelination, and neuroinflammation in HIV-1 infected brain (Figure 14). The cascade of the working model shows MBL's importance in neuronal autoimmunity in HIV-1 infected patients.

MBL mediated complement activation is involved in innate immune response in HIV-1, and HIV-1 infected brain. By MBL's binding to HIV surfactant protein gp120, opsonization of HIV-1 occurs and there is an enhancement of spreading of virus in HIV-1 infected brain. In this study, MBL has been found in major neuronal cell types such as neurons, oligodendrocytes; and myelin and increased expression of MBL with gp120 in these cell types in HIV encephalitis suggest that MBL mediated complement activation induce immune complex deposition and neuronal damage in HIV encephalitis. Furthermore, increase in proinflammatory cytokines that are related to recognition of mannose containing pathogen, which MBL interacts with, also show MBL's association with neuroinflammation in HIV+ neurocognitive impairment brain. The autoantibodies of MBL and subsequent complement proteins involved in complement activation were higher in HIV patients with neurocognitive impairment, potentially implicating the generation of autoantigens in these individuals' brain and subsequent brain damage related to excessive lectin mediated complement activation and inflammation. Higher inflammatory cytokine responses in CSF of HIV-1 infected patients with neurocognitive

impairment indicate that the presence of MBL autoantibodies is potentially associated with proinflammatory cytokine immune response in HIV related CNS impairment. These studies suggest the possibility of using MBL inhibitors for therapeutic use in HIV-1 infected neuroinflammation, neuronal damage, and neuronal autoimmunity.
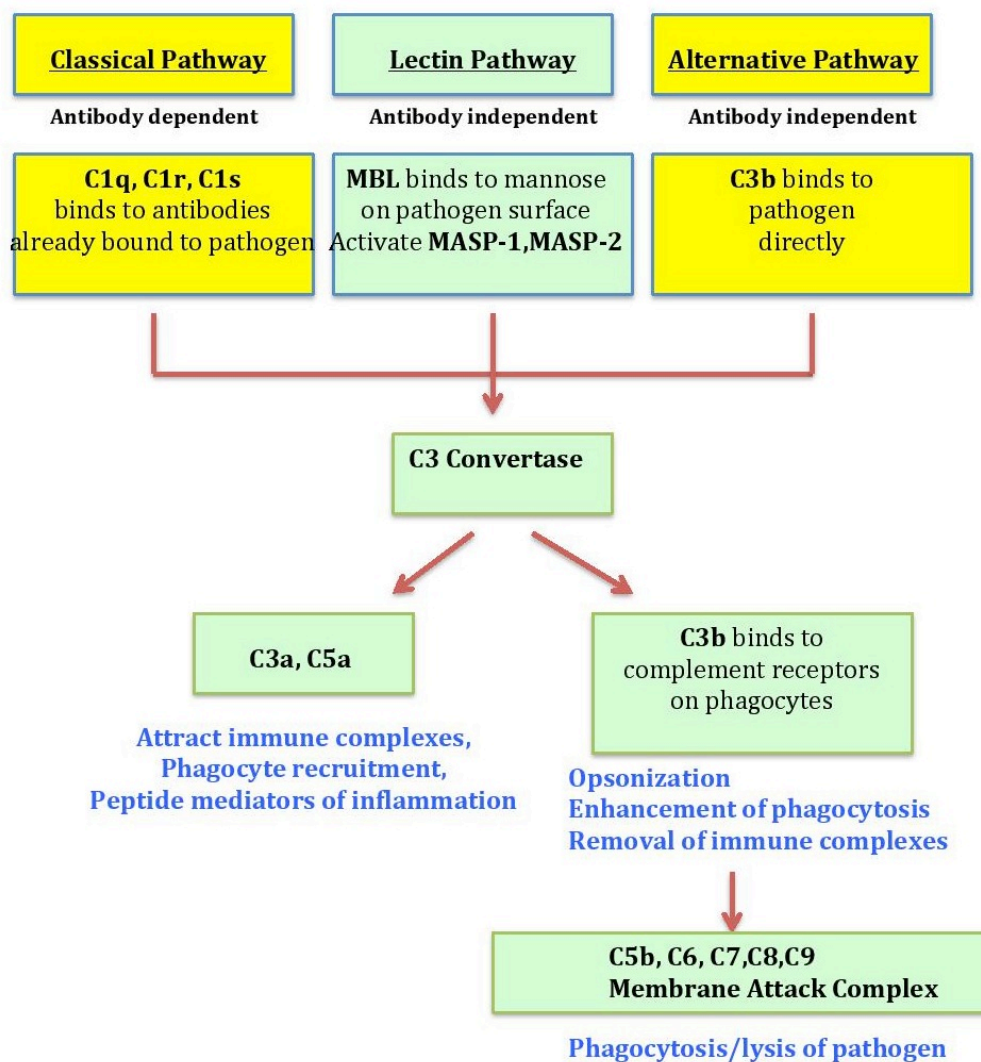
V:

FIGURES

Figure 1. Complement Activation through Classical, Lectin, and Alternative Pathway. All three pathways converge at the level of C3 convertase, and activate complement system leading to phagocytosis/lysis of pathogen.
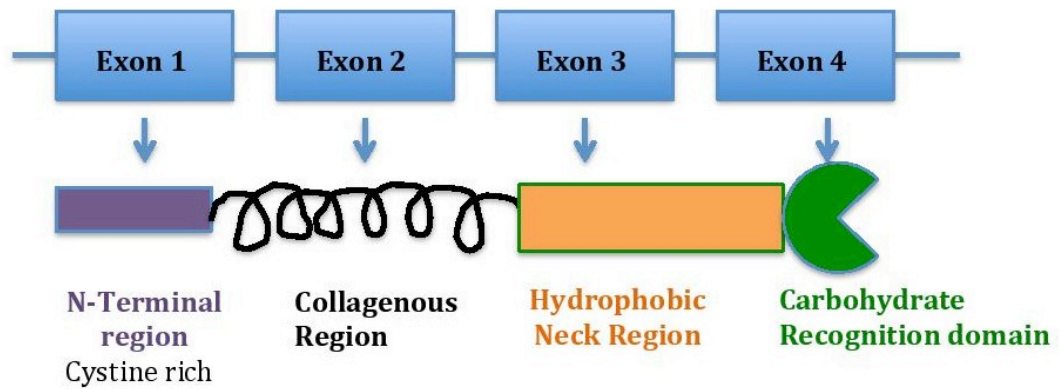
Figure 2. Structure of Mannose Binding Lectin (MBL). MBL protein is encoded from *MBL2* gene as shown above. The schematic only shows single unit of MBL, however, functional MBL is found in higher order structures.
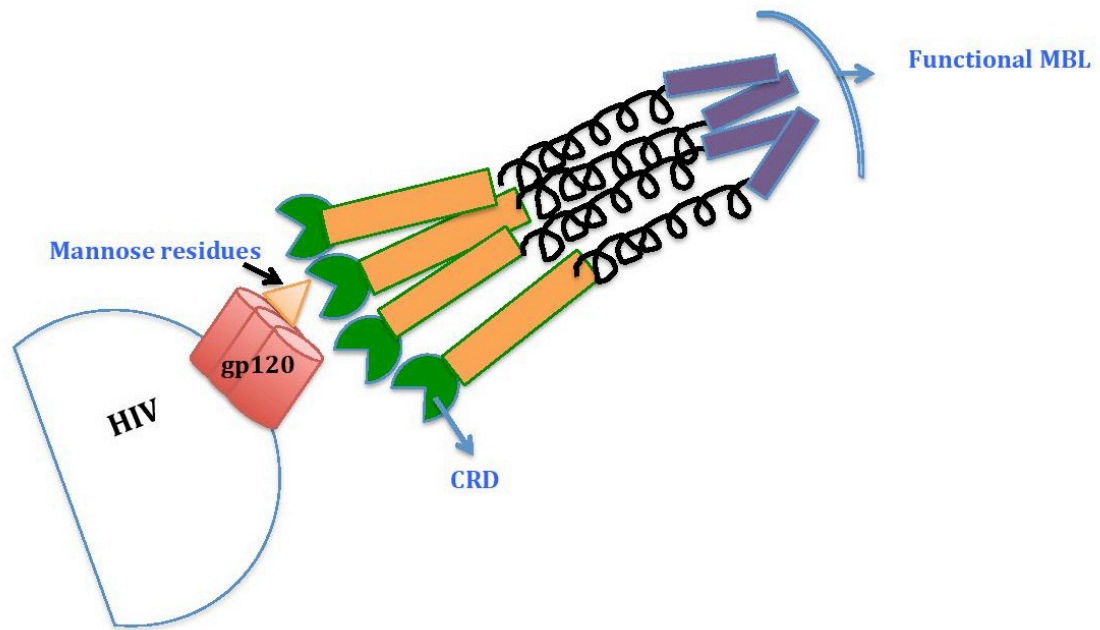
Figure 3. Schematic representation of MBL interacting with gp120 of HIV.
gp120 is present on the surface of HIV, and triangle represents high-mannose N-linked glycan. A tetramer of MBL unit is depicted as functional MBL above, and CRD stands for carbohydrate recognition domain.
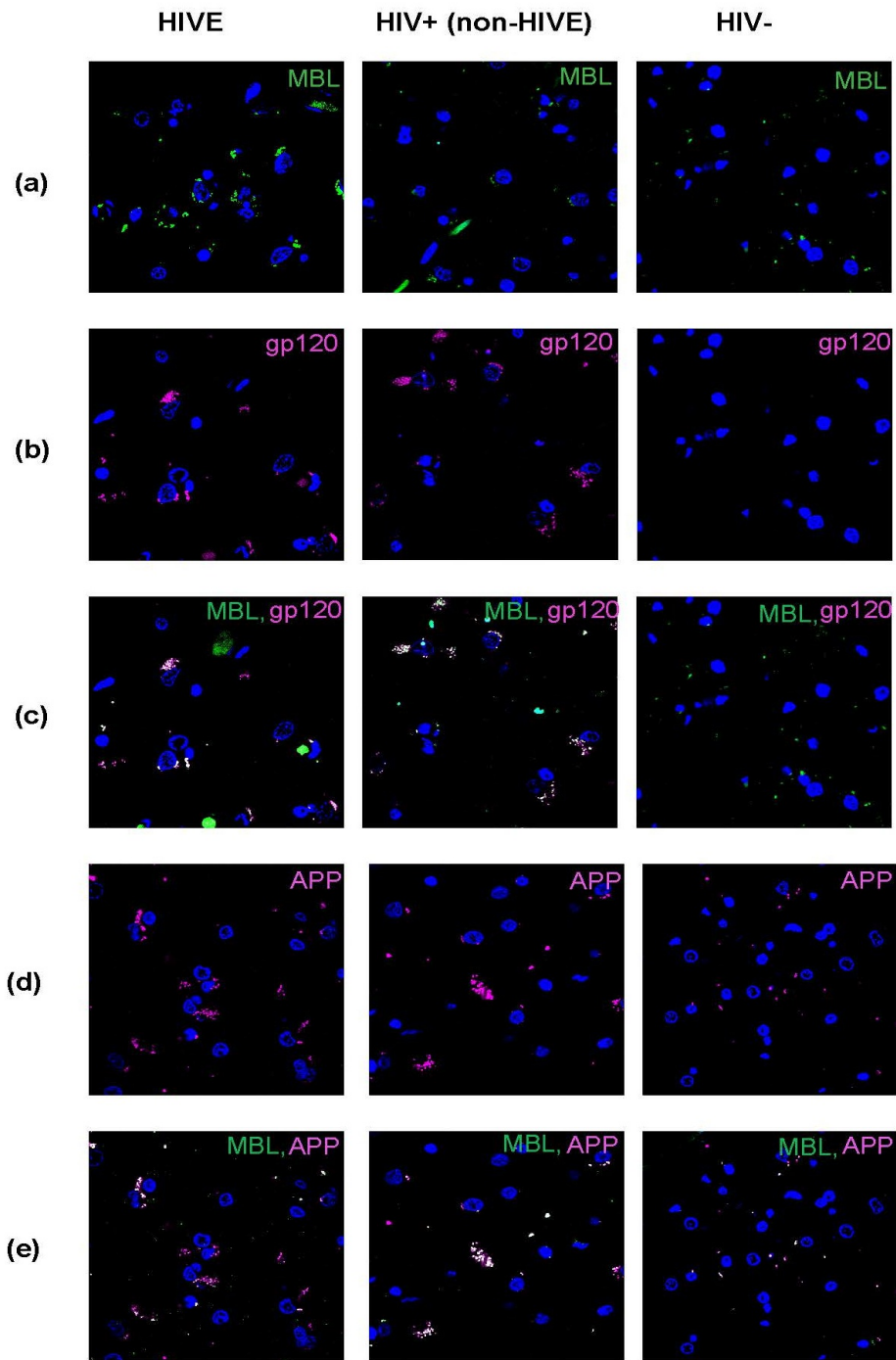
Figure 4. Immunofluorescence Analysis showing MBL with viral gp120 protein and Axonal damage marker APP. Immunofluorescence of MBL (a), gp120 (b), MBL co-localized with gp120 (c), marker for axonal damage, APP (d), and MBL co-localized with APP (e) in HIVE, HIV+ non-HIVE, and HIV- patients' frontal cortex postmortem brain tissues. DAPI, the nuclei marker is blue, MBL in green, gp120 or APP in magenta. Co-localized MBL and gp120 and/or MBL and APP are white.
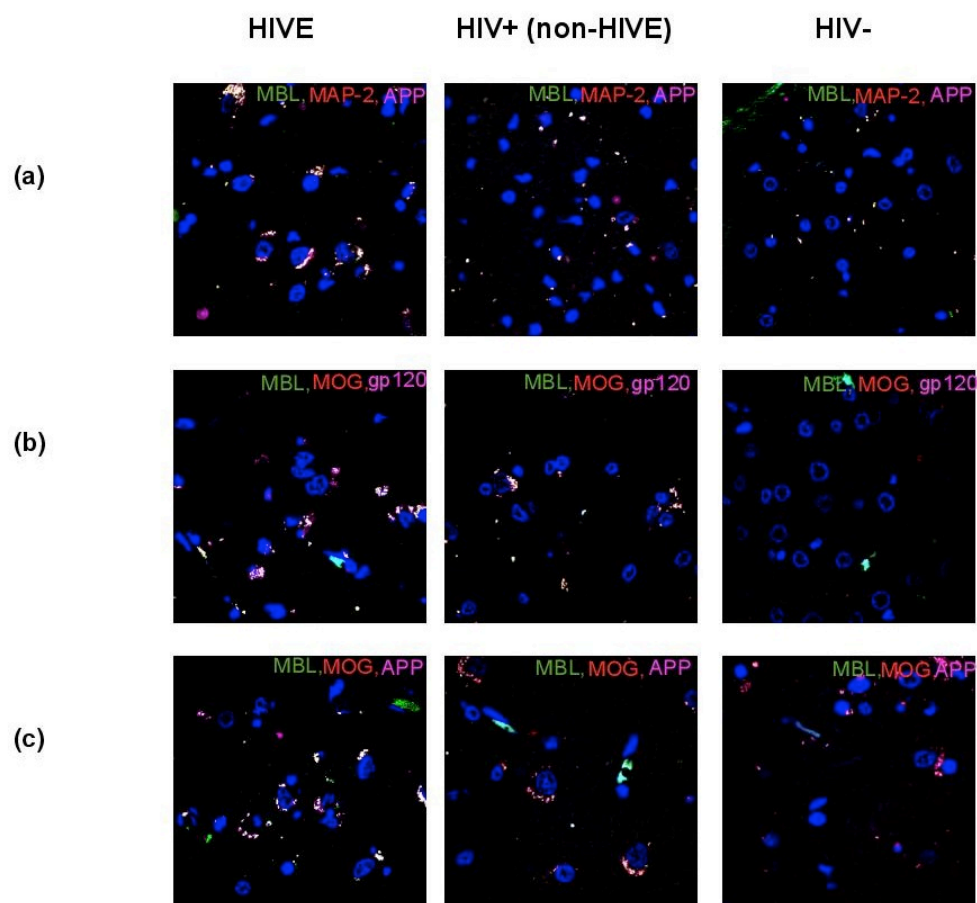
Figure 5. Immunofluorescence analysis of MBL with Neuronal Cell Marker, Oligodendrocyte marker, and Axonal Damage. Immunofluorescence of MBL co-localized with neuronal cell marker MAP-2, and gp120 (a), MBL co-localized with oligodendrocyte marker MOG, and gp120 (b), MBL co-localized with MOG, and axonal damage marker APP (c) in HIVE, HIV+ non-HIVE, and HIV- patients'' frontal cortex postmortem brain tissues. DAPI is marked in blue, MBL in green, MAP-2 and MOG in red, gp120 and APP in magenta. All three markers co-localization results in white.
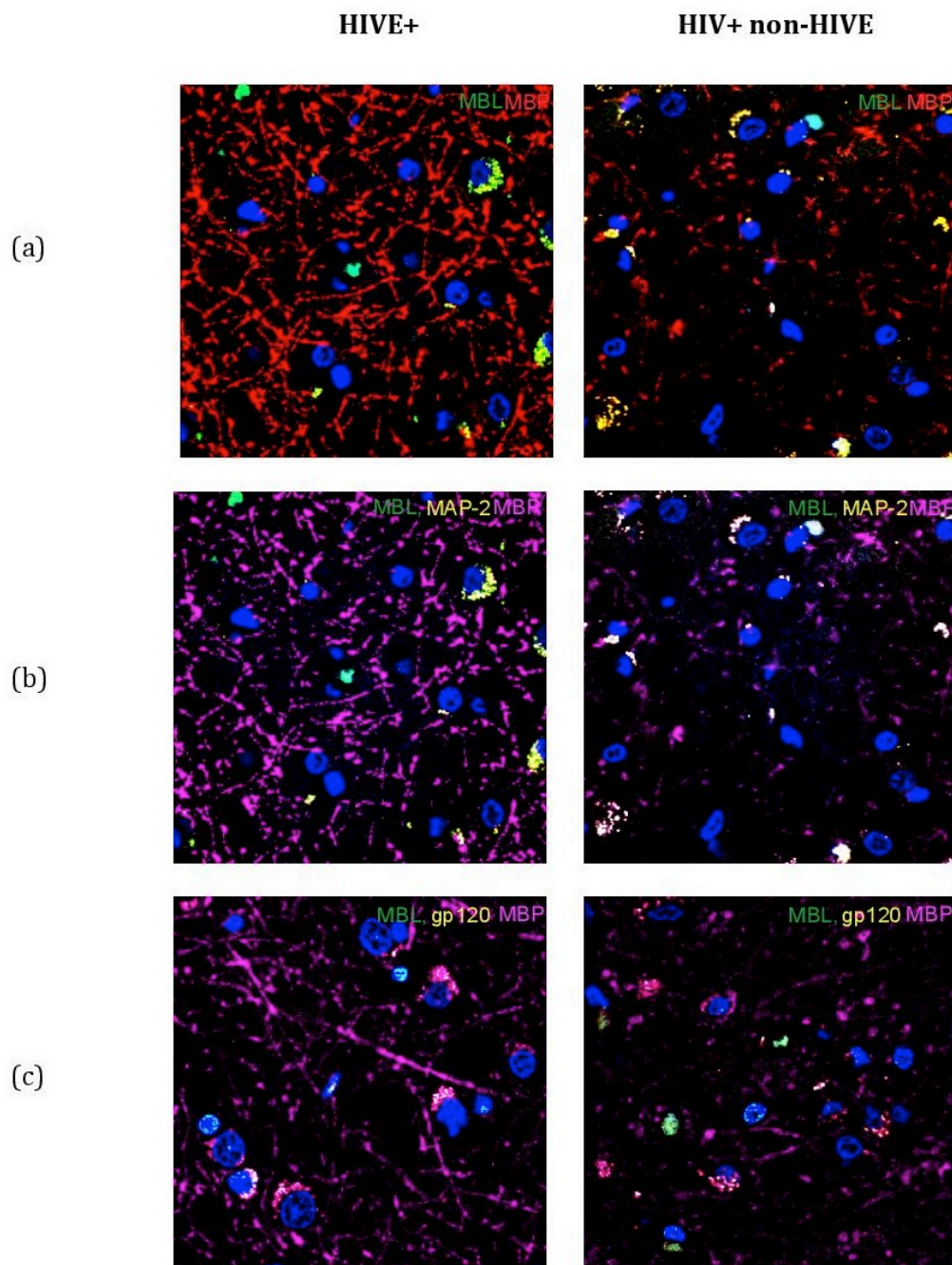
Figure 6. Immunofluorescence Analysis of MBL and Myelin Basic Protein (MBP) co-localized with neuronal cell marker MAP-2 and gp120. MBL co-localized with MBP (a), MBL co-localized with MBP and MAP-2 (b), and MBL co-localized with gp120 are shown above. DAPI is marked in blue, MBL in green, MBP in magenta, and MAP-2 and gp120 in red. All co-localization results in white.
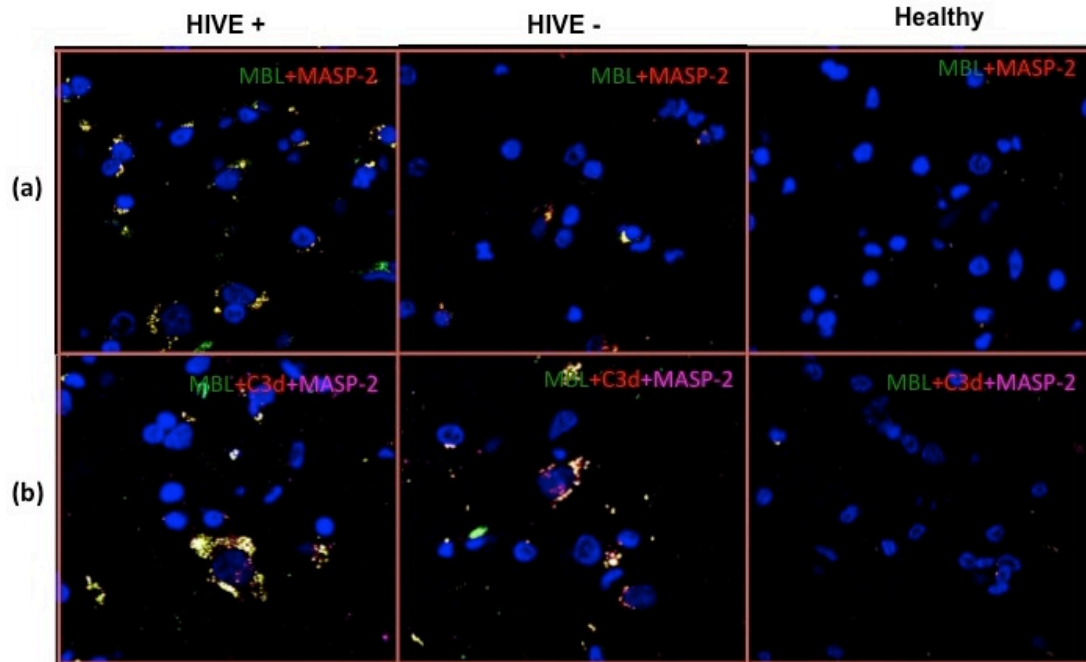
Figure 7. Increased Expression and Deposition of MBL, MASP2 and C3d Immune-complexes in HIVE Brain. MBL co-localized with MASP-2 (a), and MBL co-localized with C3d and MASP-2 (b). DAPI is marked in blue, MBL in green, MASP-2 in red (a) or magenta (b), and C3d in red. Co-localization of MBL and MASP2 is in yellow (a), and all co-localizations result in white (b).
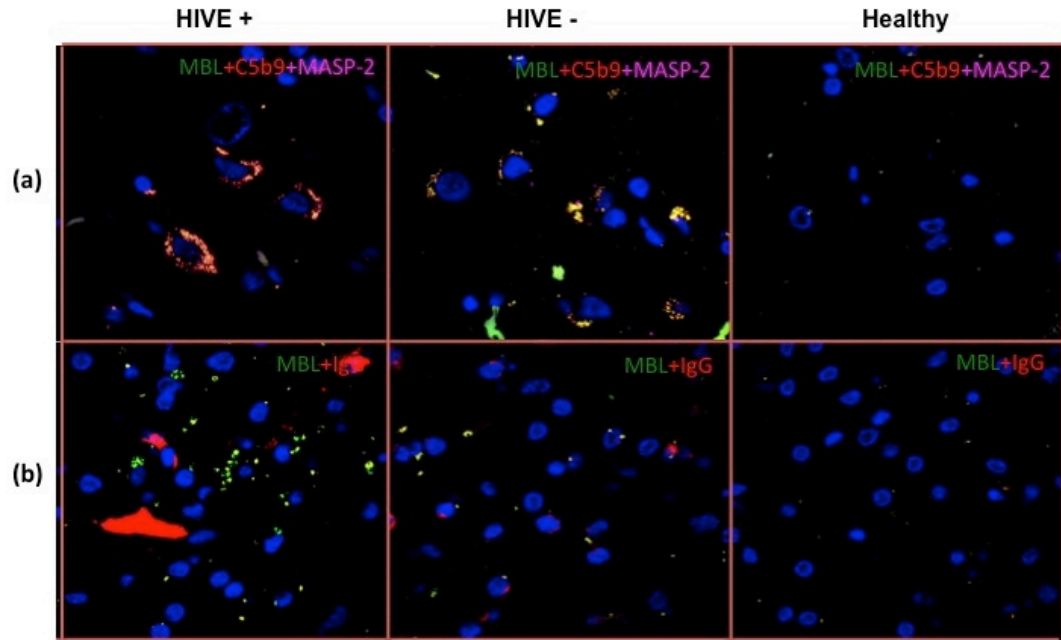
**Figure 8. Increased Expression and Deposition of MBL, MASP2 and C5b9 or MBL, IgG Immune Complexes in HIVE Brain.** Immunofluorescence analysis was performed to observe MBL co-localized with MASP-2 and C5b9 (a), and MBL co-localized with IgG (b). DAPI is marked in blue, MBL in green, C5b9 in red, MASP-2 in magenta, and IgG in red. All co-localization results in white (a), and MBL co-localizations with IgG are red (b).
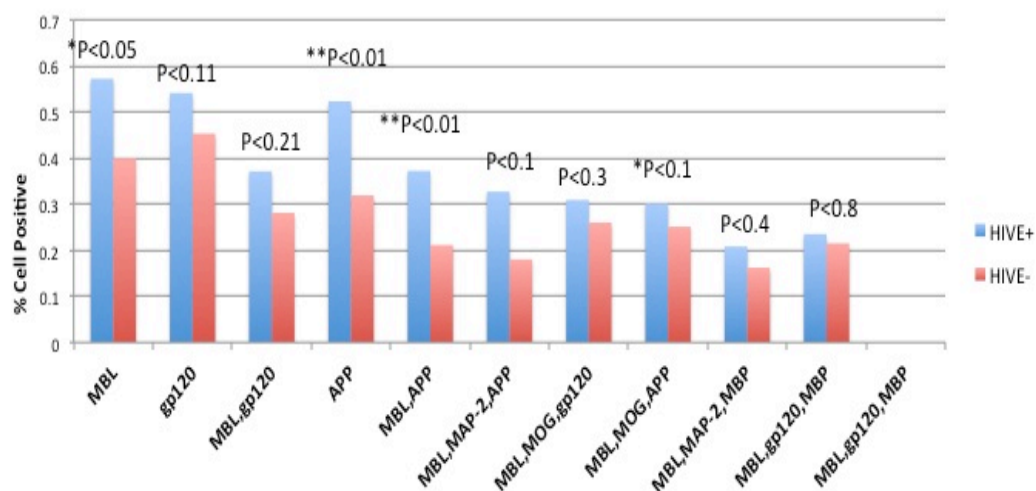
	MBL	gp120	MBL, gp120	APP	MBL, APP	MBL, MAP-2, APP	MBL, MOG, gp120	MBL, MOG, APP	MBL, MAP-2, MBP	MBL, gp120, MBP
# of HIVE+, HIVE-	(n=8,7)	(n=6,7)	(n=6,7)	(n=7,6)	(n=7,6)	(n=4,4)	(n=4,4)	(n=5,5)	(n=4,4)	(n=4,4)
Percent Increase	43%	19%	31%	65%	76%	73%	16%	43%	28%	9%

Figure 9. Summary Graph of Fold Increase of HIVE vs. non-HIVE with MBL and Viral Protein, Axonal Damage, Neuronal Cell, Oligodendrocyte, and Myelin Protein Markers. HIVE+ stands for HIV+ HIV encephalitis, HIVE- stands for HIV+ non-HIV encephalitis. Sample numbers, percent increase, and p values are shown above.

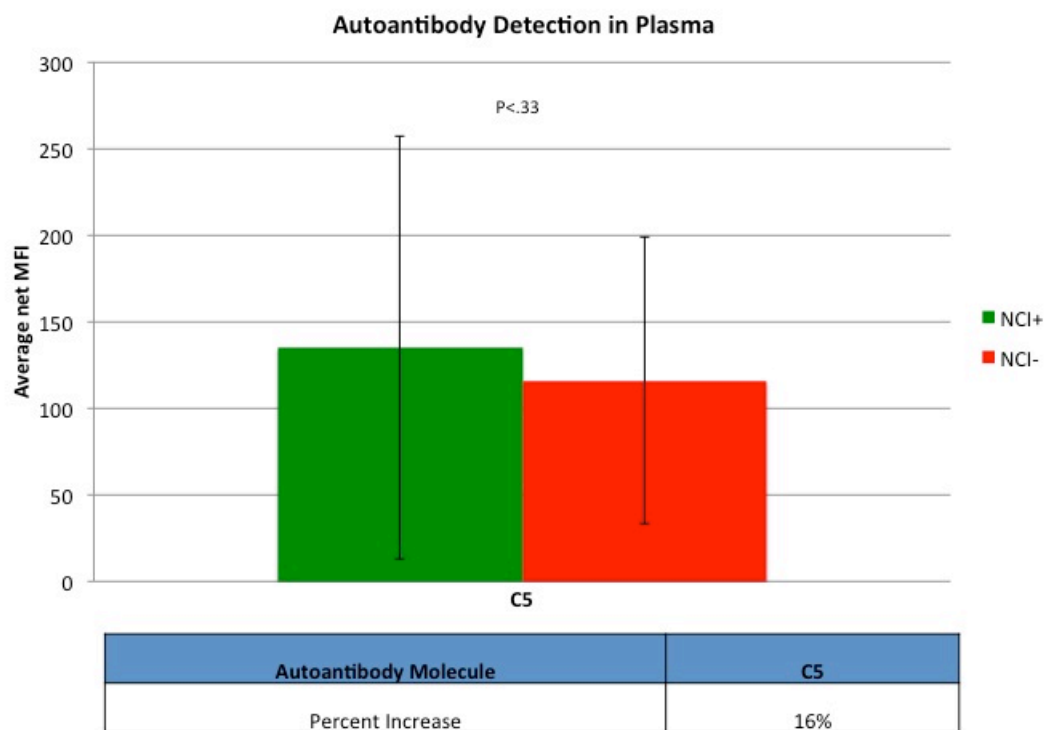**Figure 10. Higher C5 autoantibody in NCI+ vs. NCI- Plasma samples from HIV-1 infected individuals.** Autoantibody detection analysis included 20 HIV+ neurocognitive impairment + (NCI+) and 20 HIV+ and neurocognitive impairment - (NCI-) of plasma samples. Autoantibodies of C5 were shown to have significant difference between NCI+ vs. NCI-, and percent increase and p value are shown above. Error bar shows standard deviation.

Autoantibody Detection in CSF

Autoantibody Molecules	C4	C5	MBL
Percent Increase of NPI+/NPI-	13%	17%	13%

Figure 11. Higher C4, C5 and MBL Autoantibodies in Cerebrospinal Fluid of HIV-1 infected individuals with NCI. Autoantibody detection analysis included CSF samples from 19HIV+ neurocognitive impairment + (NCI+) and 20 HIV+ and neurocognitive impairment- (NCI-) cases . Autoantibodies of C4,C5, MBL had significant difference between NCI+ vs. NCI-.Percent increase and p value are shown above. Error bars show standard deviation.

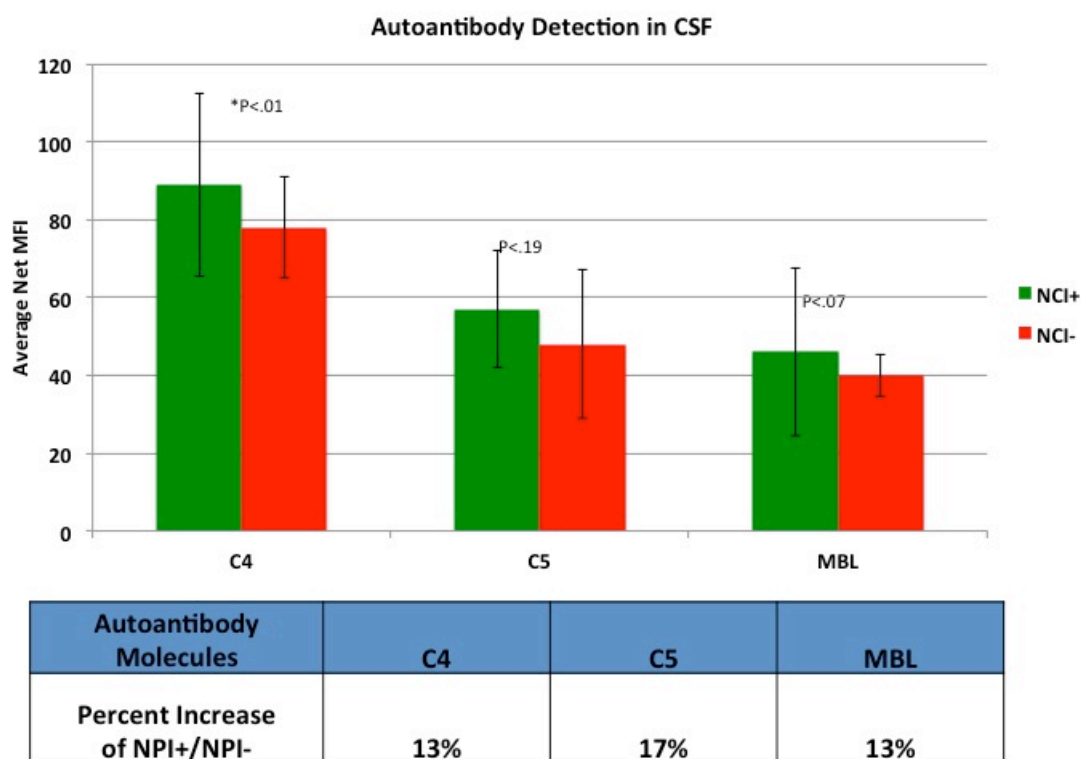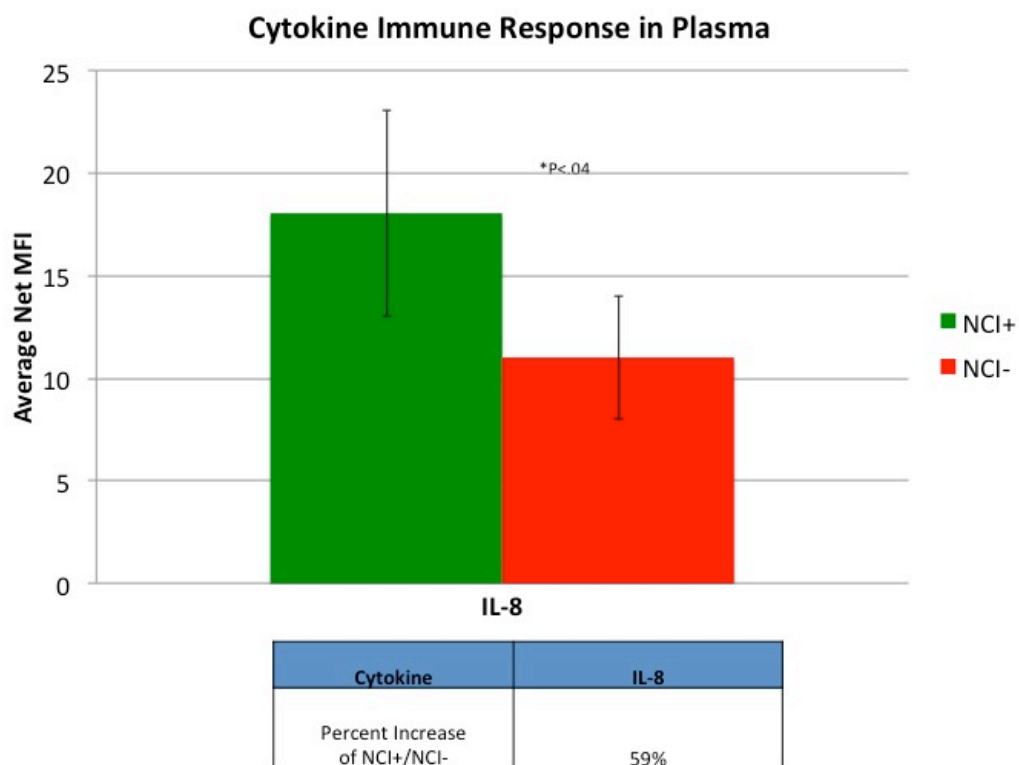**Figure 12. Higher IL-8 CytokinesIn Plasma in HIV-1 Infected Individuals with NCI.** Increase in average MFI for IL-8 is sine in NCI+, and n=11 for HIV+ NCI+, and n= 15 HIV+NCI-. Percent increase and p values are shown above. Error bars show standard deviation.

Cytokine Immune Response in CSF

Cytokine	IL-1β	IL-6	IL-2	Rantes	MIP-1β2	IL-8	MCP-1
Percent Increase of NCI+/NCI-	100%	50%	88%	44%	67%	24%	18%
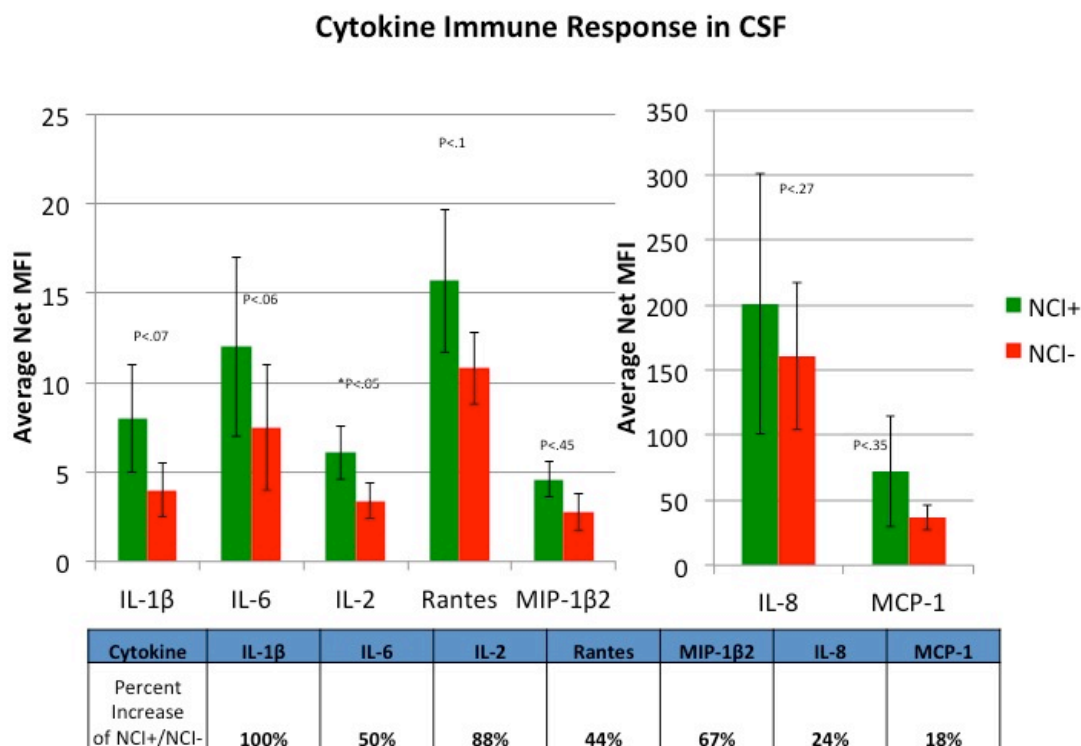
Figure 13. Higher Cytokines/Chemokines (IL-1β, IL-6, IL-2, RANTES , MIP-1β2, IL-8, and MCP-1) in CSF of HIV+ NCI+ vs. HIV+NCI-cases. For IL-1β, n= 10 for HIV+ neurocogntivie impairment, n=13 for HIV+ without neurocogntiive impairment, IL-6 (n=17 for NPI+,20 for NPI-), IL-2 (n=11 for NPI+ n=12 for NPI-), IL-8 (n=19 for NPI+,20 for NPI-), Rantes (n=10 for NPI+ n=11 for NPI-), MIP-1β2 (n=11 NPI+ n=13 for NPI-), and MCP-1 (n=16 for NPI+, n=18 for NPI-). Percent increase and p values are shown above. Error bars show standard deviation.
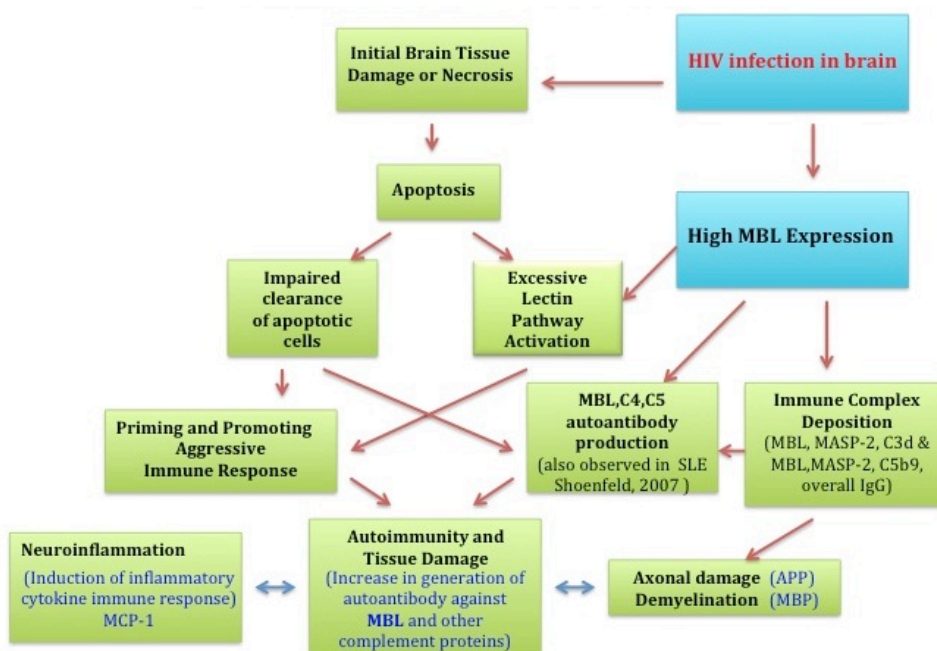
Figure 14. Working Model for the Role of the Lectin Complement Activation in Neuroinflammation, Autoimmunity/Tissue Damage, and Axonal Damage/Demyelination in HIV. Initial tissue damage/HIV Infection in brain leads to impaired clearance of apoptotic cells and excessive lectin mediated complement activation. As HIV-1 disease progresses, level of MBL increases which leads to over-activation of lectin pathway, MBL autoantibody production, and immune complex deposition. These conditions lead to neuroinflammation, neuronal damage and autoimmunity in HIV-1 infected brain.

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